Intrarectal Immunization with Rotavirus 2/6 Virus-Like Particles Induces an Antirotavirus Immune Response Localized in the Intestinal Mucosa and Protects against Rotavirus Infection in Mice‡

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Rotavirus (RV) is the main etiological agent of severe gastroenteritis in infants, and vaccination seems the most effective way to control the disease. Recombinant rotavirus-like particles composed of the viral protein 6 (VP6) and VP2 (2/6-VLPs) have been reported to induce protective immunity in mice when administered by the intranasal (i.n.) route. In this study, we show that administration of 2/6-VLPs by the intrarectal (i.r.) route together with either cholera toxin (CT) or a CpG-containing oligodeoxynucleotide as the adjuvant protects adult mice against RV infection. Moreover, when CT is used, RV shedding in animals immunized by the i.r. route is even reduced in comparison with that in animals immunized by the i.n. route. Humoral and cellular immune responses induced by these immunization protocols were analyzed. We found that although i.r. immunization with 2/6-VLPs induces lower RV-specific immunoglobulin G (IgG) and IgA levels in serum, intestinal anti-RV IgA production is higher in mice immunized by the i.r. route. Cellular immune response has been evaluated by measuring cytokine production by spleen and Peyer's patch cells (PPs) after ex vivo restimulation with RV. Mice immunized by the i.n. and i.r. routes display higher gamma interferon production in spleen and PPs, respectively. In conclusion, we demonstrate that i.r. immunization with 2/6-VLPs protects against RV infection in mice and is more efficient than i.n. immunization in inducing an anti-RV immune response in intestinal mucosa.

The induction of immune response at the level of mucosal surfaces is increasingly important in viral vaccine development. Many viruses enter the organism through mucosal tissues, and several of them also replicate in mucosal epithelial cells of the gastrointestinal and respiratory tracts. Although immunization by the parenteral route usually induces a strong systemic immune response but weak or no mucosal response, the induction of an immune response at the mucosal level often requires direct antigen application on a mucosal surface (22). The mucosa-associated lymphoid tissue represents a highly compartmentalized immunological system that functions essentially independently from the systemic immune system (22). Lymphocytes that have been primed in the mucosal inductive sites tend to migrate and exert their effector functions mainly into mucosal tissues, although not necessarily into the mucosa of origin (22). Immunization by the intranasal (i.n.) route stimulates an immune response in the respiratory tract but also in cervicovaginal mucosa, and immunization by the oral route induces antibody production not only in intestine but also in mammary glands (22). However, some compartmentalization also exists within the mucosa-associated lymphoid tissue; for instance, previous studies showed that oral immunization is

relatively inefficient for inducing an immune response in the genital tract (22).

Rotavirus (RV) is the main etiological agent of severe gastroenteritis in infants and young children worldwide, causing approximately 440,000 deaths and 1.4 billion episodes of diarrhea per year (43). Vaccination will have a significant impact on the incidence of the disease, and development of an RV vaccine remains a global priority. Since this virus essentially infects and replicates within the enterocytes of small intestine, an anti-RV immune response in the gut seems to be important to provide specific protection against RV infection. To address the safety concerns associated with live vaccines, several immunization strategies using recombinant RV proteins or non-replicating virus-like particles (VLPs) are currently being evaluated by several groups. The coexpression of the viral protein 2 (VP2) and VP6 using a recombinant baculovirus system results in their spontaneous assembly into double-layered VP2/VP6 rotavirus-like particles (2/6-VLPs) (12, 33). 2/6-VLPs have been reported to induce protective immunity in mice when administered by the oral or i.n. route together with cholera toxin (CT) or *Escherichia coli* heat-labile toxin (LT) as the adjuvant (41, 42). Immunization by the i.n. route requires smaller doses of VLPs and elicits higher serum and intestinal anti-RV antibody levels than immunization by the oral route, resulting also in a better protection against RV infection (42). However, the administration of enterotoxin-based adjuvants, such as CT and LT, by the i.n. route has recently been questioned because of the possible neurological effects that may derive from their retrograde axonal transport from the olfactory epithelium into the brain (20). Moreover, a nasal influenza

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vaccine has been withdrawn from the market recently because of the development of Bell's palsy by some recipients, and the LT present in the formulation is suspected to be responsible for this unwanted effect (39).

The intrarectal (i.r.) route for immunization has been investigated mainly for its ability to induce a local immune response on the rectal mucosa, with the aim of protecting against sexually transmitted diseases. Intrarectal immunization in mice, humans, and nonhuman primates induces high antibody titers in rectal secretions and feces (13, 23, 25, 30). Other studies showed that i.r. immunization of mice with a synthetic human immunodeficiency virus (HIV) peptide induced cytotoxic T lymphocytes (CTLs) in the spleen and in the small intestine, whereas subcutaneous immunization with the same peptide induced a CTL response only in the spleen (5, 6). In macaques, i.r. immunization with an HIV/simian immunodeficiency virus peptide is more effective against i.r. challenge with simian/human immunodeficiency virus than parenteral vaccination because of a better clearance of the virus from its major replication site in intestinal mucosa (7).

In this study we compared the protection efficacy and the immune response induced by i.r. immunization with 2/6-VLPs with those induced by i.n. immunization with the same particles. 2/6-VLPs were administered in association with two different adjuvants, CT and a CpG-containing oligodeoxynucleotide (CpG ODN), both known to be active by the mucosal route (18). CT is a widely used mucosal adjuvant which enhances both humoral and cellular immunity when administered by the oral, i.n., and i.r. routes (18, 21). CpG ODNs are synthetic oligodeoxynucleotides which contain unmethylated CpG motifs that mimic bacterial DNA and activate B cells and antigen-presenting cells by triggering Toll-like receptor 9 signaling (29). Our results demonstrate that i.r. immunization with 2/6-VLPs shows better efficacy than i.n. immunization for raising an anti-RV immune response in the digestive tract. Moreover, we observed a marked decrease of the viral shedding in mice immunized with 2/6-VLPs by the i.r. route and, when immunization is performed with CT, animals are even more protected than they are when immunized by the i.n. route.

MATERIALS AND METHODS

Materials. CT and ovalbumin (OVA) were purchased from Sigma (St. Louis, MO), and CpG ODN 1826 (sequence TCCATGA<u>CG</u>TTCCTGA<u>CG</u>TT) was synthesized with a phosphorothioate backbone by Eurogentec (Seraing, Belgium). 2/6-VLPs containing VP2 and VP6 of the RF strain of bovine RV were produced in the baculovirus expression system and purified as previously described (10, 33, 46). Protein composition was verified by Coomassie blue staining of polyacrylamide gels and by Western blot analysis using monoclonal antibodies to VP2 or VP6. No additional proteins were present. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin (BSA; Pierce) as the standard. In order to eliminate cesium chloride, 2/6-VLPs were desalted and concentrated with centrifugal filter units (Vivaspin 500; Vivascience, Hannover, Germany; cutoff, 30 kDa). Concentrated VLPs were subsequently diluted in Dulbecco's phosphate-buffered saline (D-PBS).

Viruses. The EDIM strain of murine RV was kindly provided by Javier Buesa (University of Valencia, Spain). Viral stock was prepared by orally infecting 5-day-old BALB/c suckling mice. Three days later, mice were euthanized and the whole intestinal tracts were removed, pooled, homogenized into D-PBS, aliquoted, and stored frozen until use. The 50% diarrhea-inducing dose was determined by oral inoculation of 5-day-old pups with serial 10-fold dilutions of the viral stock. The bovine RF strain of RV was propagated in the MA104 cell line

in the presence of 1 μg/ml trypsin, as previously described (34). Cell culture supernatant was subjected to three cycles of freeze-thawing, clarified by centrifugation at $400 \times g$ for 15 min, and used directly for ex vivo restimulation of spleen and Peyer's patch (PP) cells. To pellet and partially purify viral particles, supernatant from infected MA104 cells was layered onto a 40% sucrose cushion and centrifuged at $83,000 \times g$ for 90 min. The pellet was resuspended in 50 mM Tris (pH = 8.0) with 10 mM EDTA in order to remove RV outer capsid proteins. Viral antigen suspension was then diluted in 50 mM carbonate buffer (pH = 9.5), homogenized with a tissue blender to break up aggregates, and used for enzyme-linked immunosorbent assay (ELISA) plate coating.

Animals and treatments. Nine-week-old female BALB/c mice were obtained from Charles River Laboratories (L'Arbresle, France). Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies. Mice were confirmed to be RV antibody free by ELISA prior to immunization. They were immunized every other week for a total of three times with 5 μg of 2/6-VLPs together with the indicated doses of CT or CpG ODN 1826 by either the i.r. or the i.n. route. A group of control mice was mock immunized by the i.r. route with 5 μg of OVA and the indicated amount of CT. Mice were deprived of food 24 h before immunization, and during this time grids were placed on the bottoms of cages in order to prevent the animals from ingesting litter. Prior to inoculation, mice were anesthetized by intraperitoneal administration of a mixture of 80 mg of ketamine/kg of body weight (Imalgene; Merial, Lyon, France) and 16 mg of xylazine/kg (Rompun; Bayer, Puteaux, France) diluted in D-PBS in a final volume of 200 μl/mouse. For i.r. immunization, mice were given a final volume of 50 μl split in five doses of 10 μl. Immunization was performed through a 0.5- to 20-μl pipette tip (Eppendorf, Hamburg, Germany) lubricated with a thin film of petrolatum ointment and inserted approximately 1.5 to 2 cm deep. For i.n. immunization, mice were given a final volume of 20 μl split in two doses of 10 μl. Serum and stool samples were collected from each mouse 4 weeks after the last immunization. Blood was taken from retro-orbital plexus under ketamine/xylazine anesthesia, and sera were prepared. Four to 6 weeks after the last immunization, animals were subjected to oral gavage with 100 μl of intestinal homogenate from infected newborn mice containing 1,000 50% diarrhea-inducing doses of murine EDIM RV following oral administration of 100 μl of 1.33% sodium bicarbonate to neutralize stomach acidity. Fecal samples were collected from each mouse at 3, 5, and 8 days postchallenge (d.p.c.) and homogenized 1:10 (wt/vol) in PBS. The suspension was then clarified by centrifugation.

Measurement of RV-specific antibodies by ELISA. Ninety-six-well polystyrene microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl/well of partially purified RV antigen suspension (prepared as described above) diluted in carbonate buffer. Wells were washed three times with 0.05% (vol/vol) Tween 20 in PBS (PBS-T) and blocked with 200 μl of 3% BSA (Sigma) in PBS (PBS-BSA buffer) for 2 h at 37°C. After the wells were washed, 100 μl/well of serum or stool homogenate diluted in PBS-BSA was added, and plates were incubated for 90 min at 37°C. Subsequently, plates were washed and incubated (90 min at 37°C) with 100 μl/well of biotin-conjugated goat anti-mouse immunoglobulin G (IgG), IgA, IgG1, or IgG2a antibody (Southern Biotechnology, Birmingham, AL) diluted 1:5,000 in PBS-BSA. After the wash, 100 μl of horseradish peroxidase (HRP)-labeled avidin (Southern Biotechnology) diluted 1:4,000 in PBS-BSA was added to each well, and plates were incubated for 1 h at 37°C. Color was developed by adding 100 μl of Sure Blue TMB peroxidase substrate (KPL, Gaithersburg, MD), and optical density (OD) was read at 620 nm. To determine antibody titers, serum or stool homogenates were serially (threefold) diluted and the titers were calculated by determining the inverse of the dilution at an OD of 0.5.

Determination of RV antigen shedding with ELISA. Microtiter plates were coated overnight at 4°C with rat anti-SA11 immune serum diluted in carbonate buffer. Wells were washed with PBS-T and blocked with 200 μl of PBS-BSA for 2 h at 37°C. Wells were washed again and incubated for 90 min at 37°C with 100 μl/well of serial dilutions of stool homogenate in PBS-BSA. After the wells were washed, plates were incubated (90 min at 37°C) with 100 μl/well of rabbit anti-SA11 immune serum diluted in PBS-BSA. After another wash, 100 μl of HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA) diluted 1:3,000 in PBS-BSA was added to each well, and plates were incubated for 1 h at 37°C. Color was developed by adding 100 μl of TMB peroxidase substrate and OD was read at 620 nm.

Determination of RV shedding by real-time RT-PCR. Viral RNA was extracted from 20 μl of 10% stool homogenate by using a QIAamp viral RNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Viral RNA was eluted in a final volume of 80 μl of AVE buffer supplied with the kit. A single-step real-time reverse transcription (RT)-PCR was carried out by using TaqMan one-step RT-PCR master mix reagents (Applied Biosystems,

Foster City, CA) and two primers (forward, 5′-CAGCCGCAGCATGACAACT-3′; reverse, 5′-CGAATCCTGCAACCTGTATTTCT-3′) and a probe (6-carboxy-fluorescein-ACCCAGCATTCAACCACATTGTACCCATT-6-carboxytetram-ethylrhodamine) specific for a region of the VP6 coding sequence from the EDIM RV strain. RT-PCR was performed in a final volume of 50 μl containing 18 μl of purified viral RNA, 400 nM of each primer, 150 nM of probe, 25 μl of TaqMan 2× universal PCR master mix, and 1.25 μl of 40× MultiScribe and RNase inhibitor mix. A mix containing viral RNA and primers was first heated at 95°C for 5 min to denature double-stranded RNA, and then the RNA mixture was promptly chilled on ice. Master mix reagents and probe were then added, and real-time RT-PCR was performed with an ABI Prism 7700 sequence detection system (Applied Biosystems). Reverse transcription was carried out at 48°C for 30 min and followed by Taq DNA polymerase activation at 95°C for 10 min. Then, 40 PCR cycles were performed as follows: 95°C for 15 s and 60°C for 1 min. The copy number of RV genomic RNA was calculated by plotting the threshold cycle values of the samples on a standard curve generated with serial dilutions of a plasmid containing the sequence of interest. This plasmid was constructed by cloning the EDIM VP6 sequence into the pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA) and was quantified by reading absorbance at 260 nm. The theoretical detection limit of the method was estimated as around two copies per mg of stool sample.

Spleen and Peyer's patch cell cultures. PPs were carefully excised throughout the small intestinal wall. Spleen and PP single-cell suspensions were prepared by mechanical dissociation in tissue culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 50 μM 2-mercaptoethanol, and 5% heat-inactivated fetal calf serum; all were purchased from Gibco, Paisley, United Kingdom). Splenocytes and PP cells obtained from animals of the same groups were separately pooled. Cells were washed and spleen erythrocytes were removed by osmotic lysis with ACK lysing buffer (Cambrex, Walkersville, MD). Splenocytes were cultured in 24-well plates in 2 ml/well at a density of 5×10^6 cells/well, whereas PP cells were cultured in 96-well plates in 200 μl/well at 5×10^5 cells/well. Lymphocyte restimulation with RV antigen was performed by supplementing culture medium with 10% RV-infected MA104 cell supernatant. Unstimulated lymphocytes were cultured in the presence of the same amount of mock-infected MA104 cell supernatant. After 7 days of incubation at 37°C in a 5% CO_2 humidified atmosphere, supernatants were collected for cytokine quantification by ELISA.

Cytokine determination. Gamma interferon (IFN-γ) and interleukin-5 (IL-5) in cell culture supernatants were quantified by sandwich ELISA. Microtiter plates were coated overnight at 4°C with 100 μl of either rat anti-mouse IFN-γ (clone R4-6A2) or rat anti-mouse/human IL-5 (TRFK5) monoclonal antibody (BD Pharmingen, San Diego, CA) diluted in carbonate buffer at the final concentration of 2 μg/ml. Wells were washed with PBS-T and blocked with 200 μl PBS-BSA for 2 h at 37°C. Wells were washed again and incubated overnight at 4°C with 100 μl/well of splenocyte or PP cell culture supernatant. After being washed, wells were incubated for 3 h at 37°C with 100 μl of either biotin-conjugated rat anti-mouse IFN-γ (XMG1.2) or biotin-conjugated rat anti-mouse IL-5 (TRFK4) antibody (BD Pharmingen) diluted in PBS at final concentrations of 0.5 μg/ml and 1 μg/ml, respectively. After the wells were washed, 100 μl of HRP-labeled avidin (Southern Biotechnology) diluted 1:4,000 in PBS was added to each well and plates were incubated for 1 h at 37°C. Color was developed by adding 100 μl of TMB peroxidase substrate, and OD was read at 620 nm. Cytokines were quantified by using recombinant mouse IFN-γ and IL-5 (BD Pharmingen) as standards. Detection limits were around 1 ng/ml for IFN-γ and 200 pg/ml for IL-5.

ELISPOT assay for anti-RV IgA antibody-secreting cells (ASCs). Enzyme-linked immunospot (ELISPOT) assay was performed by using MultiScreen 96-well plates with Immobilon P polyvinylidene fluoride membranes (S2EMO 04 M 99; Millipore, Bedford, MA). Wells were wetted with 15 μl of 35% (vol/vol) ethanol, rinsed with PBS, and coated with 50 μl of 20 μg/ml 2/6-VLPs in PBS. After overnight incubation at 4°C, plates were washed and membranes were blocked for 4 h at 37°C with 100 μl of complete medium (RPMI 1640 supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 50 μM 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum). PP cells were then added to plates at twofold dilutions in complete medium and cultured overnight at 37°C in 5% CO_2. Plates were washed with PBS-T and incubated (3 h at 37°C) with 100 μl/well of biotin-conjugated goat anti-mouse IgA antibody (Southern Biotechnology) diluted 1:4,000 in 0.5% (wt/vol) BSA in PBS. After the plates were washed, 100 μl of streptavidin-alkaline phosphatase enzyme conjugate (Mabtech AB, Nacka, Sweden) diluted 1:1,000 in PBS was added to each well, and plates were incubated for 45 min at room temperature. After another wash, spots were developed by adding 100 μl/well of Nitro Blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrate solution (Roche Diagnostics, Penzberg, Germany). The reaction was stopped after 15 min by washing the plates with water. Wells were analyzed and spots counted by using a KS ELISPOT imaging system (Carl Zeiss, Jena, Germany).

Statistical analysis. Differences between groups were compared by Kruskal-Wallis test. A P value of <0.05 was considered statistically significant.

RESULTS

Intrarectal immunization with 2/6-VLPs induces lower anti-RV antibody levels in serum but higher anti-RV IgA in stools than intranasal immunization. To evaluate the efficacy of i.r. administration of 2/6-VLPs against RV infection, mice were immunized three times at 2-week intervals by the i.r. route with 5 μg of 2/6-VLPs together with either 10 μg of CT or 250 μg of CpG ODN 1826 as the adjuvant. Since i.n. immunization with 2/6-VLPs has been previously reported to protect mice against RV infection (41, 42), we also immunized two groups of mice by the i.n. route with the same dose of 2/6-VLPs together with either 1 μg of CT or 25 μg of CpG ODN 1826 in order to compare the immune responses induced by these two routes of immunization. A fifth group of control animals was mock immunized with 5 μg of OVA and 10 μg of CT by the i.r. route. The dose of 2/6-VLPs was determined based upon preliminary experiments (data not shown), CT was used at doses reported previously (36), and the doses of CpG ODN 1826 were adjusted similarly in order to have a 10-fold increase in the adjuvant dose utilized for i.r. immunization over that utilized for i.n. immunization.

Four weeks after the last immunization, RV-specific IgG and IgA titers were measured in serum. We found that immunization by the i.r. route induced lower IgG levels than immunization by the i.n. route when either CT or CpG ODN 1826 was used as the adjuvant (Fig. 1A). RV-specific IgA titers after i.r. immunization were also lower than those induced by i.n. immunization (Fig. 1B). No RV-specific IgG or IgA was detected in sera from OVA-immunized control mice (Fig. 1A and B). We also analyzed the relative levels of anti-RV IgG belonging to the IgG1 and IgG2a subclasses, since the IgG1/IgG2a ratio is a widely used marker to determine the type of helper T-cell response (Th2 versus Th1). Mice immunized with CT as the adjuvant by either the i.r. or the i.n. route displayed higher IgG1/IgG2a ratios than mice immunized with CpG ODN 1826 (Fig. 1C and D). These data suggest that immunization with CT as the adjuvant induces a Th2-skewed immune response, whereas immunization in the presence of CpG ODNs induces a Th1-skewed immune response, in agreement with previous reports (29, 35).

We also examined fecal antibody levels in stool samples collected 4 weeks after the last immunization. The titers of RV-specific IgA in stools were eightfold higher in mice immunized with 2/6-VLPs and CT by the i.r. route than in mice immunized by the i.n. route with the same antigen and adjuvant (Fig. 2). When CpG ODN 1826 was used as the adjuvant, i.r. immunization induced fecal IgA levels that were only twofold higher than those induced by i.n. immunization (Fig. 2).

Intrarectal immunization with 2/6-VLPs protects against RV infection in mice. In order to evaluate the protection efficacy of i.r. administration of 2/6-VLPs against RV infection, 6 weeks after the last immunization mice were orally challenged with murine RV strain EDIM, and viral shedding was mea-
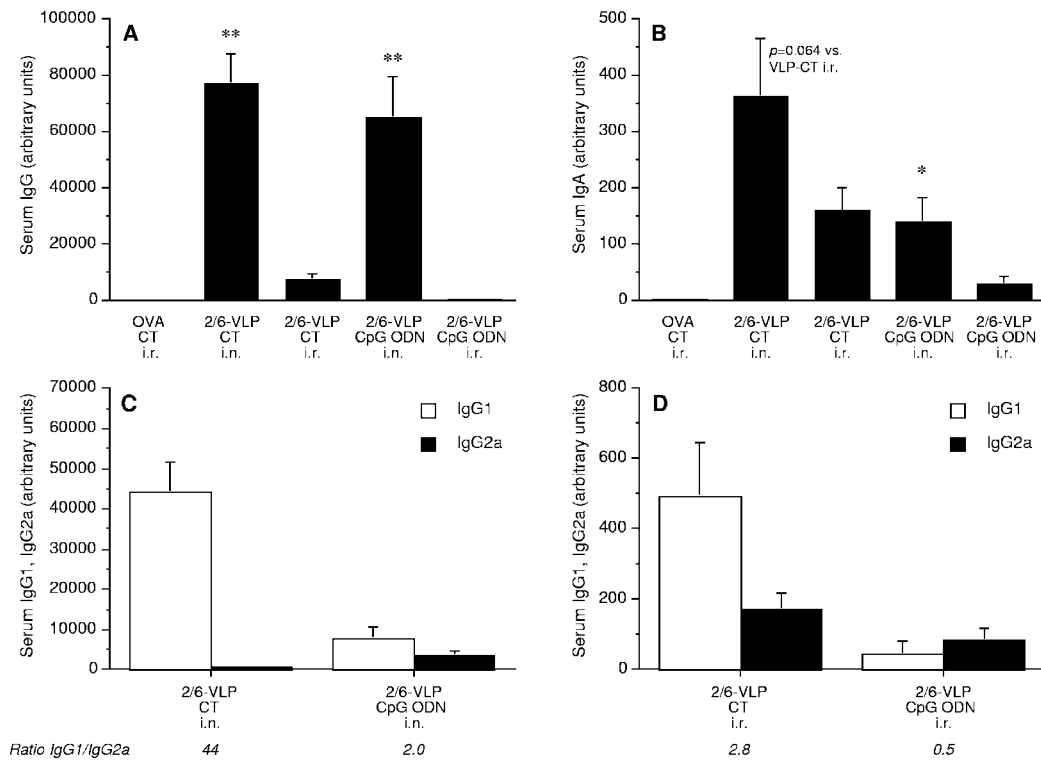
FIG. 1. Serum anti-RV antibody levels induced by i.n. and i.r. immunization with 2/6-VLPs. Mice were immunized three times at 2-week intervals with 5 μg of 2/6-VLPs in the presence of 1 μg of CT or 25 μg of CpG ODN 1826 by the i.n. route or with 5 μg of 2/6-VLPs together with 10 μg of CT or 250 μg of CpG ODN 1826 by the i.r. route. A fifth group of control mice was mock immunized with 5 μg of OVA and 10 μg of CT by the i.r. route. Four weeks after the last immunization, blood samples were taken and serum anti-RV antibodies were measured by ELISA. The levels of total anti-RV IgG (A) and IgA (B) are shown. The levels of RV-specific IgG1 and IgG2a subclasses in mice immunized with 2/6-VLPs by the i.n. (C) and i.r. (D) routes are also reported (the ratios between IgG1 and IgG2a levels are reported at the bottom of the graph). Data are displayed as mean ± standard error (SE) (six to seven mice/group). **, Significantly higher than levels of the groups of mice immunized by the i.r. route ($P < 0.01$ by the Kruskal-Wallis test). *, Significantly higher than levels of the VLP-CpG ODN i.r. group ($P < 0.05$ by the Kruskal-Wallis test).
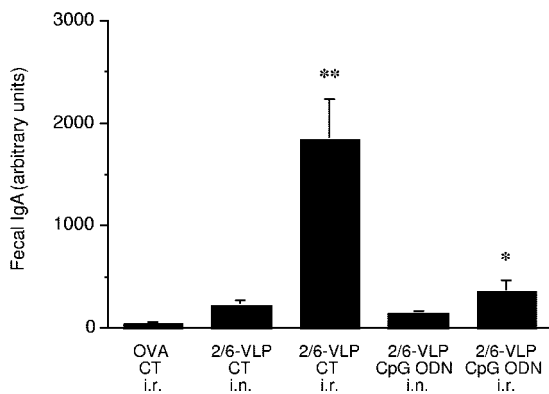

FIG. 2. Fecal anti-RV IgA titers induced by i.n. and i.r. immunization with 2/6-VLPs. Mice were immunized three times at 2-week intervals with 5 μg of 2/6-VLPs together with 1 μg of CT or 25 μg of CpG ODN 1826 by the i.n. route or with 5 μg of 2/6-VLPs together with 10 μg of CT or 250 μg of CpG ODN 1826 by the i.r. route. A fifth group of control mice was mock immunized with 5 μg of OVA and 10 μg of CT by the i.r. route. Four weeks after the last immunization, stool samples were collected and anti-RV IgA levels were measured by ELISA. Data are means ± SEs (six to seven mice/group). **, Significantly higher than levels of the VLP-CT i.n. group ($P < 0.01$ by the Kruskal-Wallis test). *, Significantly higher than levels of the VLP-CpG ODN i.n group ($P < 0.05$ by the Kruskal-Wallis test).

sured in stool samples at different time points after infection. At 3 d.p.c., RV antigen was not detected by ELISA in samples obtained from EDIM-challenged mice immunized with 2/6-VLPs and CT by either the i.n. or the i.r. route. When CpG ODN 1826 was used, stool samples were RV antigen negative for 11 of the 14 mice tested in each group (i.n. and i.r.). As expected, RV was detected in stool samples obtained from all OVA-immunized control mice (data not shown). Although these results clearly showed that i.r. immunization with 2/6-VLPs protected mice from RV infection, this evaluation of viral shedding by measuring RV antigen by ELISA in stool samples was not sensitive enough to compare the efficacies of the different immunization protocols. Moreover, RV-specific antibodies present in stool samples of immunized mice were found to compete with antibodies used for RV detection by ELISA (data not shown). Therefore, we chose to evaluate RV shedding by quantifying viral genome using real-time RT-PCR.

At 3 d.p.c., RV shedding was reduced in mice immunized with 2/6-VLPs by the i.n. route in comparison with that seen for the control mice; the RV titers decreased by 2.5 and 1.5 logs when CT and CpG ODN 1826, respectively, were used (Fig. 3A). Intrarectal immunization with 2/6-VLPs and CpG ODN 1826 led to a similar reduction in viral shedding (Fig. 3A). However, mice immunized by the i.r. route with 2/6-VLPs
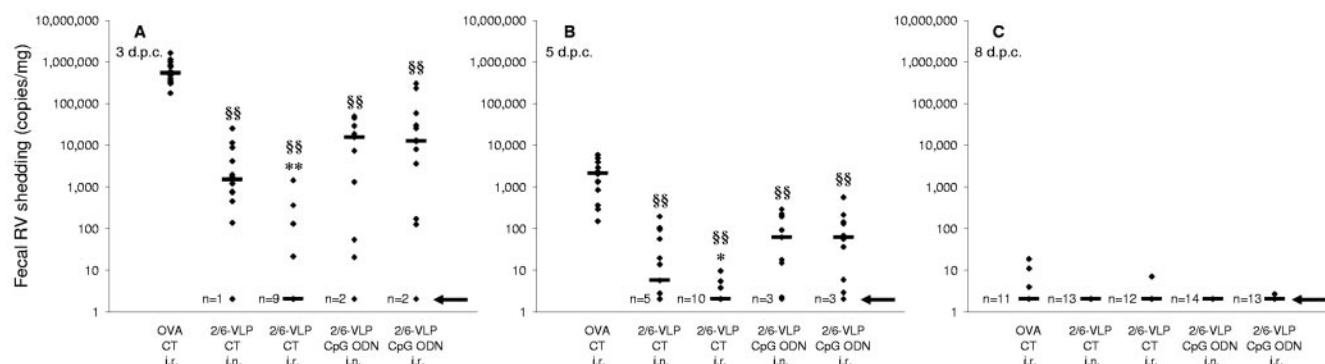
FIG. 3. Protection from RV infection in mice immunized with 2/6-VLPs by the i.n and i.r. routes. Mice were immunized three times at 2-week intervals with 5 μg of 2/6-VLPs together with 1 μg of CT or 25 μg of CpG ODN 1826 by the i.n. route or with 5 μg of 2/6-VLPs together with 10 μg of CT or 250 μg of CpG ODN 1826 by the i.r. route. A fifth group of control mice was mock immunized with 5 μg of OVA and 10 μg of CT by the i.r. route. Six weeks after the last immunization, mice were orally challenged with murine RV (strain EDIM), and viral shedding was measured in stool samples at 3 d.p.c. (A), 5 d.p.c. (B), and 8 d.p.c. (C) by quantifying genomic RNA by real-time RT-PCR. Results are given in copies of viral genome per mg of stool. Bars indicate the median RV shedding level for each group of animals (13 to 14 animals/group in two separate experiments), and diamonds indicate the amount of RV shed by each individual animal. The numbers of mice for which RV shedding was undetectable (<2 copies/mg; indicated by arrows) are also reported at the bottom of the graph. ∗∗, Significantly lower than levels of the VLP-CT i.n. group ($P < 0.01$ by the Kruskal-Wallis test). ∗, Significantly lower than levels of the VLP-CT i.n. group ($P < 0.05$ by the Kruskal-Wallis test). §§, Significantly lower than levels of the OVA-CT i.r. group ($P < 0.01$ by the Kruskal-Wallis test).

and CT displayed a higher protection against RV infection, with a 5-log reduction of the viral shedding (Fig. 3A). Within this group, viral genome was undetectable in fecal samples of 9 out of 13 mice after 40 cycles of real-time RT-PCR ($P < 0.01$ by Fisher's exact test). Conversely, RV genomes were detected in 12 out of 13 mice immunized intranasally with 2/6-VLPs and CT and in 12 out of 14 mice immunized with 2/6-VLPs and CpG ODN 1826 by either the i.n. or the i.r. route (Fig. 3A). A similar pattern was observed at 5 d.p.c., although the amounts of viral particles in the stool samples were significantly lower than those seen at 3 d.p.c. (Fig. 3B). At 8 d.p.c., RV genome was almost undetectable in fecal samples collected from any group of animals (Fig. 3C).

Intrarectal immunization with 2/6-VLPs induces lower cytokine production in spleen but higher cytokine production in PPs. We also evaluated the cellular immune response elicited by i.r. and i.n. immunization with 2/6-VLPs and the different adjuvants. Splenocytes were harvested from control and immunized mice 4 weeks after the last immunization, and production of IFN-γ and IL-5, which are Th1- and Th2-associated cytokines, respectively, was measured following ex vivo restimulation with RV. Splenocytes from mice immunized with 2/6-VLPs by the i.r. route produced less IFN-γ than splenocytes from mice immunized by the i.n. route (Fig. 4A). IL-5 production was observed only in splenocytes of mice immunized with CT, and mice immunized by the i.n. route displayed higher cytokine production than mice immunized by the i.r. route (Fig. 4B). Similar patterns of cytokine production were observed in culture supernatants of splenocytes obtained from 2/6-VLP-immu-
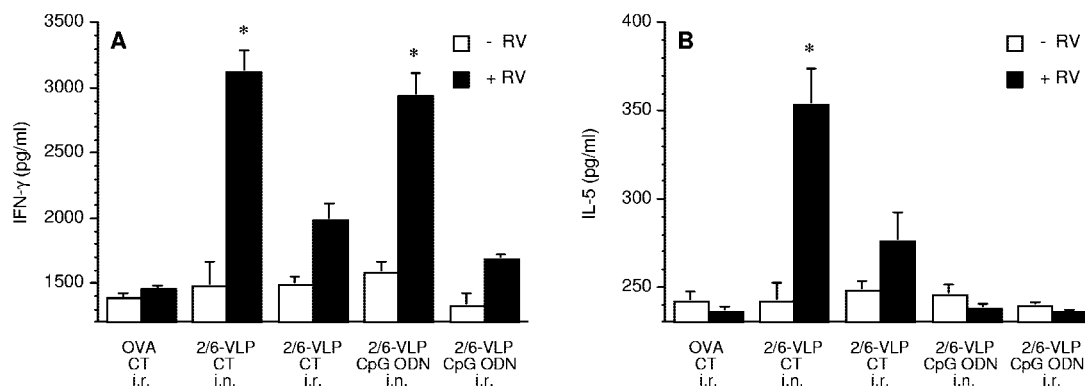

FIG. 4. Cytokine production by splenocytes of mice immunized with 2/6-VLPs by the i.n and i.r. routes. Mice were immunized three times at 2-week intervals with 5 μg of 2/6-VLPs together with 1 μg of CT or 25 μg of CpG ODN 1826 by the i.n. route or with 5 μg of 2/6-VLPs together with 10 μg of CT or 250 μg of CpG ODN 1826 by the i.r. route. A fifth group of control mice was mock immunized with 5 μg of OVA and 10 μg of CT by the i.r. route. Four weeks after the last immunization, three mice/group were sacrificed and their spleens were collected. Splenocytes obtained from mice of the same group were pooled and cultured in the presence of uninfected (−RV; white columns) or RV-infected (+RV; black columns) MA104 cell supernatant. One week later, IFN-γ (A) and IL-5 (B) levels were measured in culture supernatants by ELISA. Data are means ± SEs of three independent experiments. ∗, Significantly higher than cytokine levels produced by restimulated splenocytes from mice immunized with 2/6-VLPs and the same adjuvant by the i.r. route ($P < 0.05$ by the Kruskal-Wallis test).
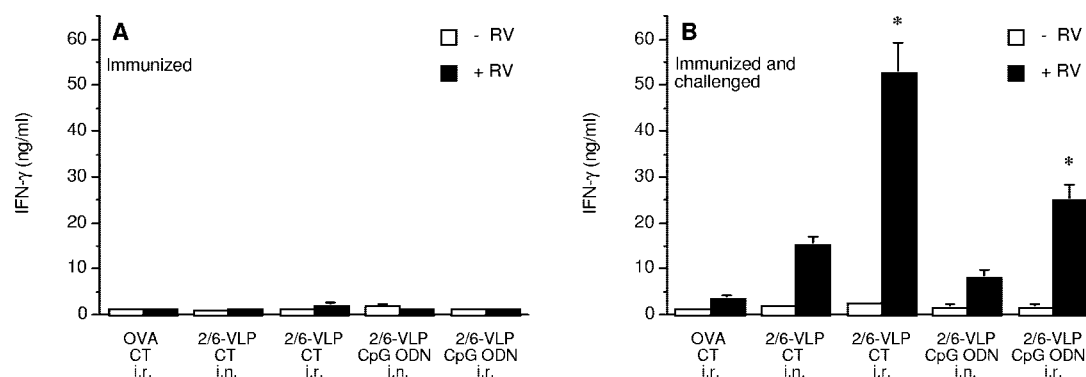
FIG. 5. Cytokine production by PP cells of mice immunized with 2/6-VLPs by the i.n and i.r. routes. Mice were immunized three times at 2-week intervals with 5 μg of 2/6-VLPs together with 1 μg of CT or 25 μg of CpG ODN 1826 by the i.n. route or with 5 μg of 2/6-VLPs together with 10 μg of CT or 250 μg of CpG ODN 1826 by the i.r. route. A fifth group of control mice was mock immunized with 5 μg of OVA and 10 μg of CT by the i.r. route. Four weeks after the last immunization, three mice/group were sacrificed and their PPs were collected. PP cells obtained from mice of the same group were pooled and cultured in the presence of uninfected (−RV; white columns) or RV-infected (+RV; black columns) MA104 cell supernatant. One week later, IFN-γ levels were measured in culture supernatants by ELISA (A). Six weeks after the last immunization, mice were orally challenged with murine RV (strain EDIM). At 3 d.p.c., animals were sacrificed and IFN-γ production by PP cells was measured as described above (B). Data are means ± SEs of three independent experiments. *, Significantly higher than IFN-γ levels produced by restimulated PP cells of mice immunized with 2/6-VLPs and the same adjuvant by the i.n. route ($P < 0.05$ by the Kruskal-Wallis test).

nized mice at 3 d.p.c. (data not shown). Neither IFN-γ nor IL-5 was observed after restimulation of splenocytes collected from OVA-immunized control mice either before infection or at 3 d.p.c. (Fig. 4 and data not shown).

Intestinal T-cell response was analyzed by measuring cytokine production by PP lymphocytes obtained from 2/6-VLPs-immunized mice before and after challenge with murine RV. No IFN-γ production was observed in culture supernatants of restimulated PP cells collected from control and 2/6-VLP-immunized mice 4 weeks after the last immunization (Fig. 5A). However, a detectable level of IFN-γ production was found in cultures of PP cells obtained from 2/6-VLP-immunized mice at 3 d.p.c.; in this case, mice immunized by the i.r. route displayed higher IFN-γ production than mice immunized by the i.n. route (Fig. 5B). No IFN-γ was observed after restimulation of PP cells collected from OVA-immunized control mice at 3 d.p.c. Low IL-5 levels were inconsistently observed after restimulation of PP cells collected at 3 d.p.c. from mice immunized with 2/6-VLPs in the presence of CT by either route but not in mice immunized with 2/6-VLPs and CpG ODN 1826 (data not shown).

Intrarectal immunization with 2/6-VLPs induces higher anti-RV IgA production in intestinal mucosa than intranasal immunization. The intestinal antibody production induced after immunization was evaluated by measuring the numbers of RV-specific IgA ASCs in PPs of immunized mice before and after challenge with murine RV. Four weeks after the last immunization, we found that ASC numbers measured by ELISPOT assay of PPs of mice immunized with 2/6-VLPs by the i.n. route were similar to the background numbers of ASCs found in OVA-immunized mice, whereas ASC numbers of PPs of mice immunized by the i.r. route were only slightly higher (Fig. 6A). At 3 d.p.c., the number of RV-specific IgA ASCs was moderately increased in PPs of mice immunized by the i.n. route, whereas a great increase in the number of ASCs was observed in PPs of mice immunized by the i.r. route with either

CT or CpG ODN 1826 as the adjuvant (Fig. 6B). No increase in the number of RV-specific ASCs was found in PPs of OVA-immunized mice at 3 d.p.c.

Intrarectal immunization with 2/6-VLPs also protects against RV infection when performed with low adjuvant doses. In order to investigate if i.r. immunization was still protective against RV infection at lower adjuvant doses, we immunized three times by the i.r. route, at 2-week intervals, several groups of mice with 5 μg of 2/6-VLPs together with 1, 3, 5, or 10 μg of CT. For comparison, other groups of mice were immunized intranasally with 5 μg of 2/6-VLPs together with 1, 3, or 5 μg of CT, and a group of control mice was left untreated (because of toxicity problems, we were not able to immunize mice with 10 μg of CT by the i.n. route). Four weeks after the last immunization, mice were orally challenged with murine RV, and viral shedding was measured in stool samples at 3 d.p.c. All the immunization protocols significantly reduced RV shedding in comparison with what was seen for the control mice (Fig. 7A). In particular, i.r. immunization with 2/6-VLPs in the presence of only 1, 3, or 5 μg of CT provided a protection against RV infection that was similar to that obtained with 10 μg of CT, with a level of viral shedding undetectable for the majority of the mice (Fig. 7A). The same levels of RV shedding were observed in mice immunized by the i.n. route in the presence of 3 or 5 μg of CT, whereas mice immunized by the i.n. route with only 1 μg of CT shed higher quantities of virus (Fig. 7A).

Cellular and humoral immune responses in the intestinal mucosa were also analyzed for mice immunized with 2/6-VLPs and 3 μg of CT by either the i.n. or the i.r. route by measuring IFN-γ or RV-specific IgA production by PP cells at 3 d.p.c. The levels of IFN-γ production after restimulation (Fig. 7B) and the numbers of RV-specific IgA ASCs (Fig. 7C) were higher in PPs from mice immunized by the i.r. route than in mice immunized by the i.n. route, indicating that i.r. immunization still induced a stronger intestinal immune response than i.n. immunization, when performed with the same dose of adjuvant.
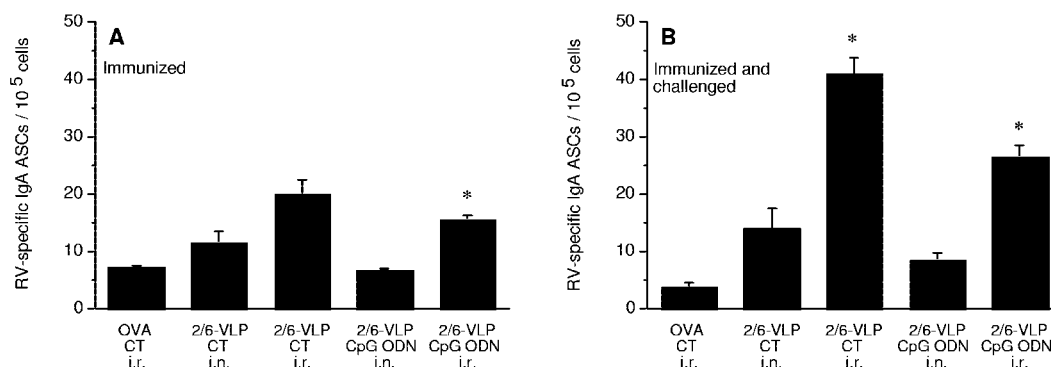
FIG. 6. Anti-RV IgA production by PP cells of mice immunized with 2/6-VLPs by the i.n and i.r. routes. Mice were immunized three times at 2-week intervals with 5 μg of 2/6-VLPs together with 1 μg of CT or 25 μg of CpG ODN 1826 by the i.n. route or with 5 μg of 2/6-VLPs together with 10 μg of CT or 250 μg of CpG ODN 1826 by the i.r. route. A fifth group of control mice was mock immunized with 5 μg of OVA and 10 μg of CT by the i.r. route. Four weeks after the last immunization, two mice/group were sacrificed and their PPs were collected. PP cells obtained from mice of the same group were pooled, and the numbers of RV-specific IgA ASCs were determined by ELISPOT assay (A). Six weeks after the last immunization, mice were orally challenged with murine RV (strain EDIM). At 3 d.p.c., animals were sacrificed and the numbers of RV-specific ASCs were determined in PPs as described above (B). Data are means ± SEs of quadruplicate wells (these experiments were repeated twice with similar results). *, Significantly higher than the ASC number found in PPs of mice immunized with 2/6-VLPs and the same adjuvant by the i.n. route ($P < 0.05$ by the Kruskal-Wallis test).

DISCUSSION

In the present study, we compared the immunogenicities and protective efficacies of 2/6-VLPs administered by the i.r. and i.n. routes in the presence of CT or CpG ODN as the adjuvant. Administration of antigens through the nasal mucosa is very efficient for the induction of an immune response and we indeed found high levels of anti-RV antibodies in sera of mice immunized with 2/6-VLPs by the i.n. route. In mice immunized by the i.r. route, the titers of RV-specific antibodies were much lower, even if immunizations were performed with higher adjuvant doses. Similar findings were reported in a human study in which i.n. administration of cholera vaccine induced higher serum antibody responses than administration by the i.r. route (31). On the contrary, when we investigated antibody production at the intestinal level, we found that i.r. immunization induces a higher number of RV-specific IgA ASCs in PPs. Similar results were found for the cellular immune responses, since mice immunized by the i.n. route displayed higher spleen IFN-γ production than mice immunized by the i.r. route, whereas mice immunized by the i.r. route displayed higher IFN-γ production by PP cells. Therefore, immunization by the i.r. route induces humoral and cellular immune responses that are localized in the intestinal mucosa. Moreover, mice immunized with 2/6-VLPs by the i.r. route are protected against RV infection, as viral shedding in stool samples was significantly reduced in comparison with that seen for OVA-immunized control animals. Furthermore, when CT is used as the adjuvant, even at low doses, mice immunized by the i.r. route show better protection than mice immunized by the i.n. route, and viral shedding is not even detectable in many of them. Hence, i.r. immunization may represent a valuable strategy for eliciting a protective immunity against intestinal pathogens such as RV.

The high levels of anti-RV IgA found in intestinal mucosa of mice immunized by the i.r. route suggest a role for these antibodies in protection against RV infection. In fact, although 2/6-VLPs do not contain the RV outer layer proteins VP4 and VP7 and therefore do not induce neutralizing antibodies, it is known that VP6-specific IgA can inhibit RV replication. Murine hybridomas producing anti-VP6 IgA implanted in a backpack model have been reported to protect adult mice from RV infection and clear chronic infection in immunocompromised mice (9). Based upon these observations, it has been postulated that anti-VP6 IgA, during its transcytosis inside enterocytes, might interact with partially decapsidated virions and inhibit viral replication. Consistently, J-chain knockout mice, defective in transcellular transport of IgA and IgM by the polymeric immunoglobulin receptor, are less protected than wild-type mice when immunized with 2/6-VLPs and CT by the i.n. route (45). However, we show that when mice are immunized with CpG ODN 1826, the intestinal production of RV-specific IgA does not correlate with the decrease in RV shedding, so other mechanisms may contribute to the protection conferred by immunization with 2/6-VLPs.

We show that i.r. immunization with 2/6-VLPs also elicits a robust T-cell response in the small intestine. Previous studies have identified a protective T-helper epitope on VP6 (3, 11), and CD4+ T cells were reported to be the only lymphocytes required to prevent RV infection in mice immunized intranasally with VP6 (38). CD4+ effector T cells are classified into two groups based upon cytokines they secrete, which are named Th1 and Th2 and promote cellular and humoral immune responses, respectively (1). The polarization of the immune response towards the Th1 or Th2 phenotype in vivo depends on several factors, and the type of adjuvant used for immunization is one of these. Adjuvants that activate Toll-like receptor signaling, such as CpG ODNs, stimulate IL-12 production by antigen-presenting cells and skew the immune response toward the Th1 phenotype (29). Immunization with CT instead has been reported to inhibit IL-12 synthesis (8) and induce a Th2-polarized or a mixed Th1/Th2 immune response (19, 35). Consistently, we found that lymphocytes from mice immunized with 2/6-VLPs together with CT produced both the Th1-associated cytokine IFN-γ and the Th2-associated IL-5. In addition, isotype analysis of anti-RV IgG antibodies shows that
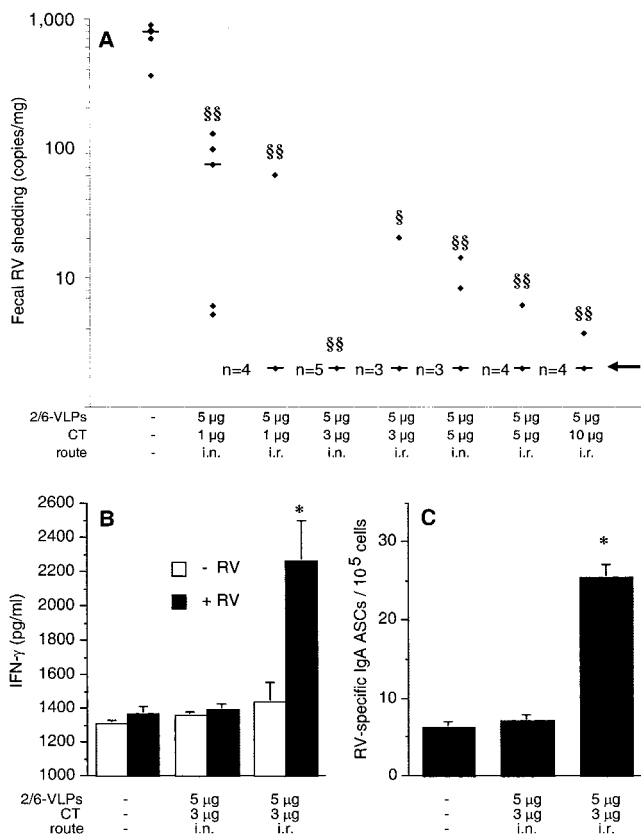
FIG. 7. Protection from RV infection and intestinal immune responses in mice immunized with 2/6-VLPs and different doses of CT by the i.n and i.r. routes. Mice were immunized three times at 2-week intervals with 5 μg of 2/6-VLPs together with 1, 3, or 5 μg of CT by the i.n. route or with 5 μg of 2/6-VLPs together with 1, 3, 5, or 10 μg of CT by the i.r. route. A fifth group of control mice was left untreated. Four weeks after the last immunization, mice were orally challenged with murine RV (strain EDIM), and viral shedding was determined in stool samples at 3 d.p.c. by quantifying genomic RNA by real-time RT-PCR (A). Results are given in copies of viral genome per mg of stool. Bars indicate the median RV shedding for each group of animals (four to five animals/group), and diamonds indicate the levels of RV shed by each individual animal. The numbers of mice for which RV shedding was undetectable (<2 copies/mg; indicated by arrows) are also reported at the bottom of the graph. At 3 d.p.c., control mice and mice immunized with 2/6-VLPs and 3 μg of CT by the i.r. or i.n. route were sacrificed and PPs removed. IFN-γ production was measured after 1 week of culturing in the presence of uninfected (−RV; white columns) or RV-infected (+RV; black columns) MA104 cell supernatant (B) and the numbers of RV-specific IgA ASCs were determined by ELISPOT assay (C). Data shown in panels B and C are means ± SEs of quadruplicate wells. § and §§, Significantly lower than levels of control mice (P < 0.05 and P < 0.01, respectively, by the Kruskal-Wallis test). *, Significantly higher than ASC numbers or IFN-γ levels produced by PP cells of mice immunized by the i.n. route (P < 0.05 by the Kruskal-Wallis test).

in mice immunized in the presence of CT, the IgG1/IgG2a ratio is high, confirming that CT skews the effector response towards Th2. On the contrary, lymphocytes from mice immunized with CpG ODN 1826 produced only IFN-γ, and the IgG1/IgG2a ratio is low, indicating that CpG ODN 1826 skews the response towards Th1. The Th1-associated cytokine IFN-γ is well known for its important immunomodulatory and also

antiviral activity but does not seem to play a major role in the response against RV. Indeed, IFN-γ$^{-/-}$ mice clear RV infection as efficiently as wild-type mice and are equally protected by i.n. immunization with VP6 and attenuated LT (2, 17, 47). Conversely, cytokines produced by Th2 lymphocytes may enhance the humoral anti-RV response, for instance, intestinal IgA production. IL-5 is known to induce maturation of IgA-committed B cells into IgA-producing plasma cells (4).

It is known that CD8$^+$ T cells also play an important role in RV clearance in mice (15, 37). VP6 is one of the targets of CTLs, and several CD8$^+$ T-cell epitopes are harbored by this protein (16, 24), indicating that the anti-RV immune response induced by immunization with 2/6-VLPs may be partly CTL mediated. Indeed, both CT and CpG ODNs have been reported to facilitate the development of antigen-specific CTLs (6, 29); therefore, the role played by these lymphocytes in our immunization protocols needs further investigation.

It seems that the local microenvironment where T and B cells are primed with antigen determines their homing preferences; for instance, the activation of naive lymphocytes in lymphoid tissues draining the intestinal mucosa leads to the generation of effector and memory cells that migrate selectively into the gut. This tissue-selective trafficking of lymphocytes is related to the expression of specific adhesion molecules and chemokine receptors that recognize ligands which are selectively expressed in these tissues. The homing of T cells and IgA-producing plasma cells to the small intestine depends on the expression of the integrin α$_4$β$_7$ and the chemokine receptor CCR9 (32). The α$_4$β$_7$ integrin interacts with the mucosal addressin cell adhesion molecule MAdCAM-1, which is expressed on intestinal endothelium, whereas CCR9 is the receptor for chemokine CCL25, which is abundantly produced by epithelial and endothelial cells of small bowel (32). Immunization by the oral and i.r. routes in humans has been shown to induce ASCs which mostly express α$_4$β$_7$ integrin, whereas only a minor fraction of them express L-selectin, a homing receptor that confers tissue specificity for peripheral lymph nodes (27, 44). ASCs induced by i.n. immunization display a mixed pattern of these adhesion molecules, and most of them express both α$_4$β$_7$ and L-selectin (27, 44). More recently, a study with mice showed that i.n. immunization with 2/6-VLPs induced a high frequency of RV-specific B cells in the respiratory lymphoid tissue and spleen, but only a minor fraction of them expressed the α$_4$β$_7$ integrin (40). Taken together, these results may explain why the administration of antigens by the i.n. route evokes an immune response that is mainly systemic but rather weak in the gut (32). On the other hand, our data suggest that lymphocytes activated by i.r. immunization migrate to the intestine, and further studies are required to determine which pattern of adhesion molecules is expressed by T and B cells primed in the rectal mucosa.

Studies conducted with humans and nonhuman primates showed that i.r. immunization, in the absence of infection, could induce high ASC numbers in rectum but low ASC numbers in duodenum and jejunum, suggesting that the compartmentalization of the immune system may differ among species (14, 26). However, i.r. immunization in macaques with the HIV/simian immunodeficiency virus peptide has been reported to clear viral load not only in colon but also in jejunum after challenge with simian/human immunodeficiency virus (7). It

should also be noted that in mice immunized with 2/6-VLPs by the i.r. route, the numbers of RV-specific ASCs in PPs are relatively low in the absence of infection, but they rapidly increase after RV administration, suggesting that at the intestinal level i.r. immunization primes an immune response that can be boosted by the challenge. Similarly, i.r. immunization with recombinant urease from *Helicobacter pylori* has been reported to induce detectable numbers of antigen-specific ASCs in stomach only after bacterial challenge (28).

In conclusion, we have shown here that in mice, i.r. immunization with 2/6-VLPs induces a powerful anti-RV immune response in gut and protects against RV infection. Therefore, i.r. immunization may be an efficient way to direct the immune response towards the small bowel, offering the same advantages as oral immunization but avoiding immunogen degradation by gastrointestinal enzymes. These results might be important for the development of vaccines not only against RV but also against other viruses that replicate in the bowel mucosa.

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